Specific Modification of Two Tryptophans within the Nuclear-Encoded Subunits of Bovine Cytochrome *c* Oxidase by Hydrogen Peroxide[†,‡]

Andrej Musatov,*[,§] Emmy Hebert, Christopher A. Carroll, Susan T. Weintraub, and Neal C. Robinson

Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900

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ABSTRACT: Hydrogen peroxide does more than react with the binuclear center of oxidized bovine cytochrome *c* oxidase and generate the well-characterized "peroxy" and "ferryl" forms. Hydrogen peroxide also inactivates detergent-solubilized cytochrome *c* oxidase in a time- and concentration-dependent manner. There is a 70−80% decrease of electron-transport activity, peroxidation of bound cardiolipin, modification of two nuclear-encoded subunits (IV and VIIc), and dissociation of ∼60% of subunits VIa and VIIa. Modification of subunit VIIc and dissociation of subunit VIIa are coupled events that probably are responsible for the inactivation of cytochrome *c* oxidase. When cytochrome *c* oxidase is exposed to 500 μM hydrogen peroxide for 30 min at pH 7.4 and room temperature, subunits IV (modified up to 20%) and VIIc (modified up to 70%) each have an increased mass of 16 Da as detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and electrospray ionization mass spectrometry. In each case, the increased mass is caused by oxidation of a tryptophan (Trp19 within subunit VIIc and Trp48 within subunit IV), almost certainly due to formation of hydroxytryptophan. We conclude that hydrogen peroxide-induced oxidation of tryptophan and cardiolipin proceeds via the binuclear center since both modifications are prevented if the binuclear center is first blocked with cyanide. Bound cardiolipin and oxidized tryptophans are localized relatively far from the binuclear center (30−60 Å); therefore, oxidation probably occurs by migration of a free radical generated at the binuclear center to these distal reaction sites.

Cytochrome *c* oxidase (CcO)[1] (EC 1.9.3.1) is the terminal complex of the inner mitochondrial electron-transport chain. This multisubunit enzyme catalyzes the four-electron reduction of dioxygen to water, a reaction coupled to proton translocation across the inner mitochondrial membrane. The monomeric unit of the enzyme contains four redox active centers, including cytochromes *a* and a_3 and the Cu_A and Cu_B centers. Electrons are transferred sequentially from ferrocytochrome *c* to Cu_A, to cytochrome *a*, and then to the binuclear center (cytochrome a_3 and Cu_B) where oxygen reduction takes place.

The mechanism for O_2 reduction by CcO involves formation of several intermediates, including the "peroxy" (P or 607 nm) and the "ferryl" (F or 580 nm) forms (*1*, *2*). It is generally accepted that CcO species related to these intermediates can be experimentally produced by addition of H_2O_2 (*3*, *4*). Low concentrations of hydrogen peroxide (micromolar) produce the peroxy form at basic pH (*5*, *6*); higher concentrations (millimolar) produce the ferryl form (*4*, *6*). H_2O_2-treated CcO is usually a mixture of these two forms although the ferryl form predominates at acidic pH (*7*).

Hydrogen peroxide can also cause structural and/or functional changes to CcO. Free radicals and superoxide are generated during the reaction of H_2O_2 with the binuclear center of CcO (*7*−*12*), both of which could oxidize amino acids within CcO. Furthermore, amino acid radicals and superoxide anion could peroxidize CL that is bound to CcO.

Bovine heart CcO is a protein−phospholipid complex consisting of 13 dissimilar protein subunits with a combined molecular mass for monomeric CcO of 205000 Da (*13*). The three largest subunits (I, II, and III) are encoded on the mitochondrial genome and include all of the redox-active centers. The remaining 10 "small" subunits are nuclear encoded and most likely have a structural and/or regulatory role (*14*, *15*). For example, removal of subunit VIa prevents CcO dimerization (*16*), removal of subunit VIIa causes a coincident and complete loss of CcO electron-transport activity (*17*), and modification of subunit VIIc and/or subunit VIII with 4-hydroxy-2-nonenal, a major lipid peroxidation product, also inhibits CcO (*18*). The four cardiolipins bound to purified CcO serve both a structural and functional role since complete cardiolipin removal results in decreased CcO

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* To whom correspondence should be addressed. E-mail: musatov@uthscsa.edu. Telephone: (210) 567-3753. Fax: (210) 567-6595.

[§] On leave from the Institute of Experimental Physics, Košice, Slovakia.

[1] Abbreviations: CcO, bovine heart cytochrome *c* oxidase; SMP, submitochondrial particles; TFA, trifluoroacetic acid; TBA, thiobarbituric acid; DM, dodecyl maltoside; MnSOD, manganese superoxide dismutase; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; HPLC, high-performance liquid chromatography; ESI/MS, electrospray ionization mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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electron-transport activity and dissociation of subunits VIa and VIb (*19*, *20*). Peroxidation of these essential cardiolipins might be expected to alter the activity and/or structure of CcO. In fact, peroxidation of mitochondrial membrane CL does decrease the electron-transport activity of CcO (*21*).

In this work, we examined the effect of H_2O_2 on structure and enzymatic activity of purified, detergent-solubilized CcO. We have shown that, in addition to generating the well-known peroxy and ferryl forms, reaction of H_2O_2 with CcO leads to a number of irreversible structural and functional alterations.

EXPERIMENTAL PROCEDURES

Materials. Manganese superoxide dismutase, bovine liver catalase, sodium cyanide, sodium cholate, and mannitol were all purchased from Sigma Chemical Co. Hydrogen peroxide (30% w/v of H_2O_2 in water) and malonaldehyde bis(dimethyl acetal) were from Aldrich Chemical Co., Inc. Dodecyl maltoside was purchased from Anatrace Inc. PD-10 columns (Sephadex G-25M) were purchased from Amersham Pharmacia Biotech AB. Sequencing grade modified trypsin was from Promega. All other chemicals were of analytical grade.

Cytochrome c Oxidase. Bovine heart CcO (a modified Fowler-type preparation) was isolated from heart muscle particles as previously described (*22*). The final enzyme precipitate was solubilized in 0.1 M NaH_2PO_4, pH 7.4, buffer, containing 25 mM sodium cholate and 1.0 mM EDTA. The enzyme preparation (~120 μM heme aa_3) was frozen and stored in liquid nitrogen. The purified enzyme contained 9.5−10 nmol of heme *a*/mg of protein and had a molecular activity of 350−370 s^{-1} when assayed spectrophotometrically with 30 μM ferrocytochrome *c* as a substrate at pH 7.0 (*22*).

Methods. CcO was reacted with H_2O_2 at room temperature in the presence of 2 mM sodium cholate and 2 mM dodecyl maltoside, conditions that produce stable dimeric CcO (*16*). The concentration of H_2O_2 was determined using $\epsilon_{240nm} = 40\ M^{-1}\ cm^{-1}$ (*23*). The reaction was stopped either by dilution or by removing excess H_2O_2 by anion-exchange (HiTrapQ) or PD-10 (Sephadex G-25M) column chromatography. Subunits dissociated from H_2O_2-modified CcO also were removed by HiTrapQ FPLC ion-exchange chromatography. Visible spectra were collected using an SLM Aminco 3000 diode array spectrophotometer. Cyanide-inhibited CcO for reaction with H_2O_2 was prepared by reacting of 5 μM CcO with 5 mM KCN for 18 h at 4 °C. Formation of the cyanide−CcO complex was confirmed by spectroscopic analysis.

Cardiolipin Peroxidation Assays. Two methods were used to detect peroxidation of CcO bound CL. (i) UV spectral analysis of HPLC purified CL: Cardiolipin was extracted from control and H_2O_2-treated CcO by the method of Bligh and Dyer (*24*), the extract dried under N_2, and the residue dissolved in the HPLC mobile phase (cyclohexane/2-propanol/5 mM aqueous phosphoric acid in water, 50:50:2.9, v/v/v) and purified by silicic acid HPLC (*25*). The UV spectrum (205−300 nm) of CL eluting from the column was monitored using a Waters diode array detector. CL peroxidation was monitored by the appearance of conjugated dienes ($\lambda_{max} = 234$ nm), as described by Buege and Aust (*26*). (ii) Detection of thiobarbituric acid (TBA) reactive substances: CL was extracted from control and H_2O_2-treated CcO, solubilized in 50 mM Tris−SO_4 buffer containing 2 mM dodecyl maltoside, and reacted for 20 min with 0.5% 2-thiobarbituric acid dissolved in 1.4% (w/v) trichloroacetic acid. The concentration of TBA-reactive substances was determined by measuring the absorbance at 534 nm using MDA as a standard. MDA was prepared from malonaldehyde bis(dimethylacetal) by hydrolysis in 0.05 N HCl. MDA concentrations were determined using $\epsilon_{267nm} = 31500\ M^{-1}\ cm^{-1}$ (*27*).

Subunit and Cardiolipin Stoichiometry within CcO. The content of the 10 nuclear-encoded subunits within control and H_2O_2-treated CcO was quantified using C_{18} reversed-phase HPLC as previously described (*28*). The CL content of these preparations was determined by phosphorus analysis after perchloric acid wet ashing of Bligh and Dyer extracted phospholipids (*24*).

Mass Spectrometry. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were acquired on an Applied Biosystems Voyager-DE-STR operated in either the linear or reflectron mode. Matrices (dissolved in 50% acetonitrile/0.1% TFA) included sinapinic acid (50 mg/mL)/dodecyl maltoside (2 mg/mL) for intact subunits, 2,5-dihydroxybenzoic acid (50 mg/mL) for tryptic digests, and 2,5-dihydroxybenzoic acid (50 mg/mL)/citric acid (100 mM) for CL. Spectra represent the average of 100 laser shots. HPLC−electrospray ionization tandem mass spectra were obtained using a Finnigan LCQ in conjunction with a Michrom BioResources MAGIC 2002 micro-HPLC connected to a home-built microspray interface. HPLC conditions were as follows: column, New Objective PicoFrit (75 μm i.d., 15 μm tip) packed to 10 cm with Vydac 218MSB5 (5 mm, 300 Å); mobile phase A, 0.5% acetic acid, 0.005% TFA in water; mobile phase B, 0.5% acetic acid, 0.005% TFA, 90% acetonitrile in water; gradient, 2% mobile phase B to 72% mobile phase B in 30 min; flow rate, 0.4 μL/min. MS conditions: ESI voltage, 3 kV; isolation window for HPLC−ESI/MS/MS, 3; isolation window for infusion−ESI/MS/MS, 2; collision energy, 35%.

RESULTS

Spectral Changes of Cytochrome c Oxidase Induced by Hydrogen Peroxide. Reaction of CcO with H_2O_2 at neutral pH produced the expected absorption spectral changes in the Soret and α-region similar to those previously described for detergent-solubilized CcO (*7*) (Figure 1). The Soret maximum shifted from an initial value of 419.0 nm to 426.4 nm after 30 min incubation with 1 mM H_2O_2 (Figure 1, inset). In addition, the maximum absorbance intensity decreased by about 10% (Figure 1, inset). As expected, the difference spectrum in the Soret area did not have a defined isosbestic point, was asymmetric, and was characterized by a time-dependent shift in the maximum from 436 to 438 nm (Figure 1, main panel). The α-region was characterized by two maxima, one at 580 nm and the other at 607 nm, consistent with the production of a mixture of the ferryl and peroxy forms (*7*).

Inactivation of Cytochrome c Oxidase by Hydrogen Peroxide. The molecular activity of H_2O_2-treated CcO decreased by approximately 70% after incubation with 1 mM H_2O_2 for 30 min (Figure 2). Inactivation was pseudo first order because semilogarithmic plots of CcO inactivation

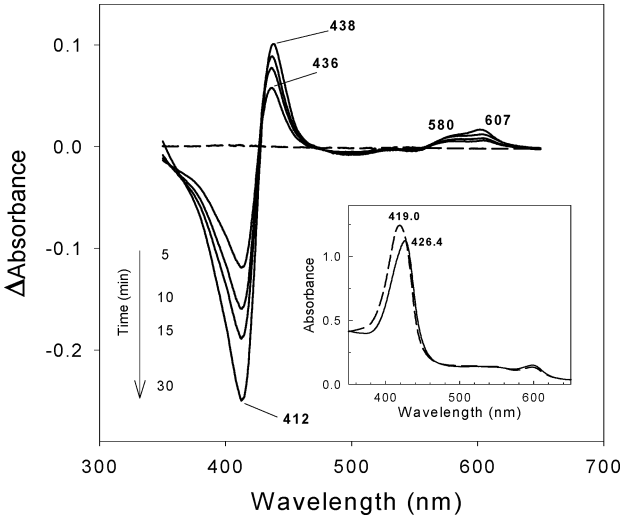

FIGURE 1: Time-dependent spectral changes of cytochrome *c* oxidase induced by hydrogen peroxide. Main panel: Difference spectra of 8 μM CcO reacted with 1 mM H_2O_2 for 5, 10, 15, and 30 min. The dashed line is the baseline with CcO in both cuvettes before addition of H_2O_2. CcO was solubilized in 20 mM Tris−SO_4 buffer containing 2 mM sodium cholate and 2 mM dodecyl maltoside, pH 7.2. Inset panel: Absolute spectra before (dashed line) and after (solid line) reaction of CcO with 1 mM H_2O_2 for 30 min.

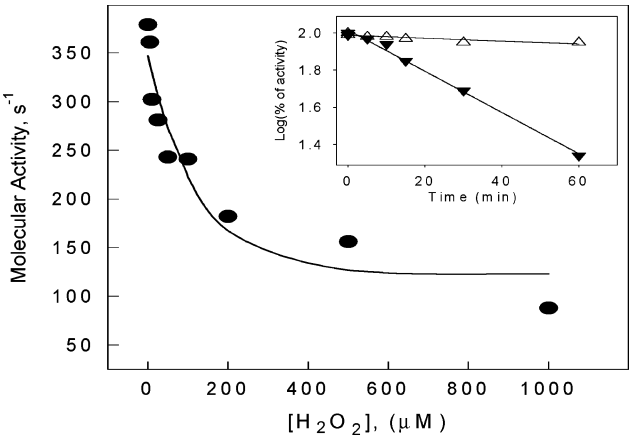

FIGURE 2: Hydrogen peroxide inactivation of cytochrome *c* oxidase. Main panel: Hydrogen peroxide concentration-dependent loss of CcO electron-transport activity. CcO (5 μM) was reacted with each concentration of H_2O_2 for 30 min; excess H_2O_2 was removed by gel filtration chromatography. Untreated CcO remained fully active over this time period. Data were fitted to a single-exponential decay (solid line). Inset panel: Semilogarithmic plot of activity versus time for CcO (5 μM) reacted with 5 μM H_2O_2 (open triangles) and 500 μM H_2O_2 (filled triangles). Reaction with H_2O_2 was stopped by gel filtration chromatography prior to activity assay.

versus time of incubation were linear with 5−1000 μM H_2O_2 (Figure 2, inset). Electron-transfer activities were measured after the reactions were stopped by dilution or by removal of excess H_2O_2 using either anion-exchange or PD-10 column chromatography. Isolation of H_2O_2-treated CcO by either method led to partial reversal of the H_2O_2-induced spectral changes as evidenced by a 2−3 nm blue shift in the Soret band. Because activity was not restored when the peroxy and ferryl forms were converted to oxidized CcO, we conclude that inactivation was not correlated with the formation of either intermediate but must be due to damage to either the protein or phospholipid content of CcO.

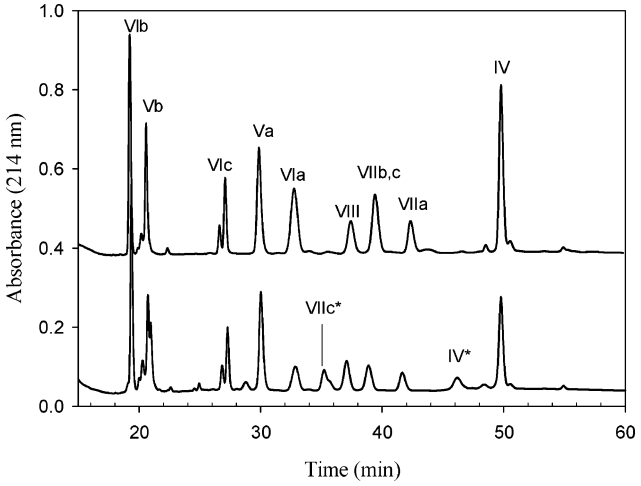

FIGURE 3: Modification of CcO nuclear-encoded subunits quantified by reversed-phase HPLC. CcO (5 μM) was incubated with and without 500 μM H_2O_2 in 20 mM Tris−SO_4, pH 7.2, buffer containing 2 mM sodium cholate and 2 mM dodecyl maltoside for 30 min. The reaction was stopped, and excess H_2O_2 was removed by PD-10 gel filtration chromatography. The elution profile of untreated CcO (upper chromatogram) is compared with the elution profile of H_2O_2-treated CcO (lower chromatogram). The two H_2O_2-modified subunits are indicated with an asterisk.

RP-HPLC Quantitative Analysis of H_2O_2-Modified Cytochrome c Oxidase Nuclear-Encoded Subunits. All 10 nuclear-encoded subunits were present in H_2O_2-treated CcO after the reaction was stopped and excess peroxide removed by gel filtration chromatography (Figure 3, lower chromatogram). However, the areas of the RP-HPLC peaks corresponding to subunits VIa and VIIa were decreased by 50−60%, indicating partial dissociation of these two subunits. Two new RP-HPLC peaks with retention times of approximately 35 and 46 min also were detected, suggesting that two subunits were modified by H_2O_2. There were no observable changes in any of the other nuclear-encoded subunits.

The absorbance intensity of each new peak was H_2O_2 concentration and time dependent. The area under each of the two peaks (subsequently shown to be modified subunit VIIc and IV by mass spectrometry; see below) was quantified and plotted as the percent of subunit modification as a function of time (Figure 4). Modification of each subunit progressed in a time-dependent manner with a maximum of 70% for subunit VIIc and 20% for subunit IV. Similarly, the percent formation of the new products increased as the H_2O_2 concentration was increased from 5 to 500 μM (data not shown).

Identification of the Two H_2O_2-Modified CcO Subunits by Mass Spectrometry. The modified subunit that eluted with a retention time of approximately 35 min had a molecular mass 16 Da greater than subunit VIIc. This is consistent with the addition of one oxygen atom (Figure 5). The modified subunit that eluted with a retention time of approximately 46 min had a molecular mass close to that of subunit IV. However, the accuracy of MALDI-TOF/MS in this mass range (17153 Da) is not sufficient to determine if one or two oxygen atoms had been inserted. ESI/MS/MS was later used to confirm that subunit IV had been chemically modified by the insertion of one oxygen atom (see below).

ESI/MS/MS was used to identify Trp19 as the site modified within subunit VIIc. The approach was analogous to that employed to identify the site of HNE modification

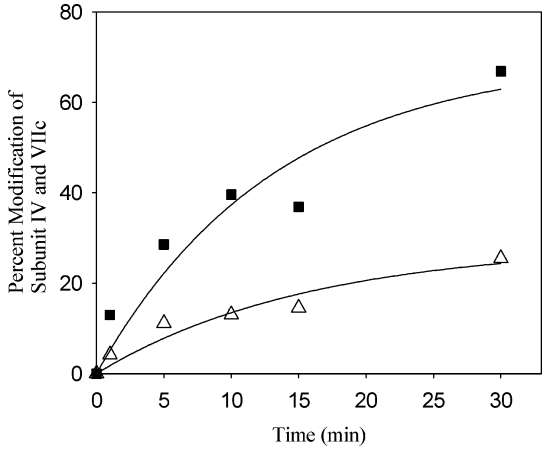

FIGURE 4: Time-dependent modification of subunit IV and subunit VIIc. CcO (5 μM) was reacted with 500 μM H_2O_2, the reaction stopped at each time by PD-10 gel filtration chromatography, and the H_2O_2-modified CcO analyzed by RP-HPLC (refer to Figure 3). Areas of elution peaks corresponding to modified subunit IV (triangles) and modified subunit VIIc (squares) were measured and plotted versus time. The percent modification of each subunit was calculated on the assumption that the extinction coefficient at 214 nm was the same for H_2O_2-modified and unmodified subunits. Differences between chromatograms were normalized on the assumption that the area under the RP-HPLC peak corresponding to subunit Va remained constant.

within CcO subunit VIII (*18*). Unmodified and H_2O_2-modified subunits VIIc were each isolated by RP-HPLC and infused directly into the ion trap mass spectrometer. Ions representing charge states from 4+ to 7+ were detected for both samples, yielding deconvoluted molecular masses of 5440 Da (control) and 5456 Da (H_2O_2-treated). MS/MS spectra were then generated for the 4+, 5+, and 6+ ions in each sample by collision-induced dissociation in the ion trap. The resulting spectra indicated the addition of an oxygen atom to Trp19. The close agreement of a series of C-terminal fragments ($y_{12}-y_{26}$) in both samples clearly eliminated His42 and the C-terminal half of the subunit (from Leu22 to Lys47) as the site of modification. The numerous identical b-series ions in the range of b_3-b_{16} for the control and H_2O_2-treated samples likewise indicated that the oxygen atom was not attached to His2. Comparison of the $b_{23}-b_{35}$ and $y_{29}-y_{44}$ ions in both samples provided strong evidence for attachment of one oxygen atom to Trp19.

The site of oxygen addition within subunit VIIc was unambiguously identified by analysis of tryptic peptides by MALDI-TOF/MS and HPLC-ESI/MS/MS. Analysis of the uninterpreted MS/MS data by the Mascot software package (Matrix Science) indicated that Trp19 was oxidized within modified subunit VIIc. Assignments for the CID fragment ions in the spectra, obtained from the tryptic peptide containing Trp19 (NIPFSVENKWR), were made for both samples by comparison with the theoretical fragments generated in silico by GPMAW (Lighthouse Data). Clearly, Trp19 was modified by the addition of a single oxygen atom during peroxide treatment (Table 1).

The site of modification within subunit IV was also determined by analysis of tryptic peptides using MALDI-TOF/MS and HPLC-ESI/MS/MS. Addition of one oxygen atom to Trp48 occurred, as is evident from the summary of the CID fragments generated from CcO subunit IV peptide 46−56 (ASWSSLSIDEK) (Table 2).

Peroxidation of Cardiolipin Associated with Detergent-Solubilized CcO. The cardiolipin content of CcO was unaffected by its exposure to 1 mM H_2O_2 for 30 min; 4 mol of cardiolipin remained bound per mole of CcO. However, the H_2O_2 reaction did cause cardiolipin peroxidation as was evident from the generation of TBA-reactive substances and conjugated dienes. The concentration of TBA-reactive substances increased 2-fold (Figure 6) and correlated with CcO inactivation. Cardiolipin extracted from H_2O_2-treated CcO also contained conjugated dienes, which is consistent with cardiolipin peroxidation. However, the extent of cardiolipin damage was relatively small since MALDI-TOF/MS analysis only detected unmodified cardiolipin with a molecular mass of 1448 Da (data not shown). There was no evidence of a cardiolipin species with smaller mass; therefore, acyl chain cleavage was, at most, only a few percent.

Effect of Cyanide, Superoxide Dismutase, and Mannitol on H_2O_2-Induced Damage to CcO. H_2O_2-induced changes in CcO could not be prevented by scavengers of either hydroxyl radicals or superoxide anions. Inclusion of mannitol and/or MnSOD during the reaction of CcO with H_2O_2 did not prevent either subunit modification or CcO inactivation. However, H_2O_2-induced spectral changes, subunit modification, and cardiolipin peroxidation were completely prevented if the binuclear center was first blocked by cyanide (data not shown).

DISCUSSION

The reaction of hydrogen peroxide with CcO is much more complicated than just the well-documented generation of the peroxy and ferryl catalytic intermediates. Hydrogen peroxide causes a number of irreversible changes within CcO including loss of electron-transport activity, peroxidation of tightly bound cardiolipin, partial loss of subunits VIa and VIIa, and oxidation of two tryptophan residues (Trp19 within subunit VIIc and Trp48 within subunit IV). None of these modifications occur via direct reaction of CcO with H_2O_2 since they do not occur if the binuclear center is first blocked by cyanide. We, therefore, conclude that these reactions are initiated by the reaction of H_2O_2 with the binuclear center.

The most likely mechanism for oxidation of tryptophan or cardiolipin involves the following: (1) reaction of H_2O_2 with CcO at the binuclear center, (2) generation of a free radical, and (3) subsequent free radical migration to the sites of oxidative damage, i.e., tryptophan or bound cardiolipin. The migration is long range since distances between heme a_3 and Trp19 of subunit VIIc and Trp48 of subunit IV are 44 and 62 Å, respectively (*13*). Similarly, the distance between the binuclear center and bound cardiolipin is quite large: >40 Å to cardiolipin bound near subunit VIa and >30 Å to cardiolipin bound near subunits VIIa and VIIc. There is precedent for such long-range free radical migration as it occurs within a variety of proteins and peptides, including CcO (*9*). The pathway for radical migration within CcO is not known, and analysis of the bovine CcO 3-D structure (*13*) did not reveal an obvious aromatic pathway. However, the structure suggested the existence of a narrow hydrophobic channel between the binuclear center and Trp19 of subunit VIIc. This channel is through subunit I, passes the isoprenoid tail of cytochrome *a*, and is surrounded by primarily hydrophobic amino acids, i.e., methionines, isoleucines,

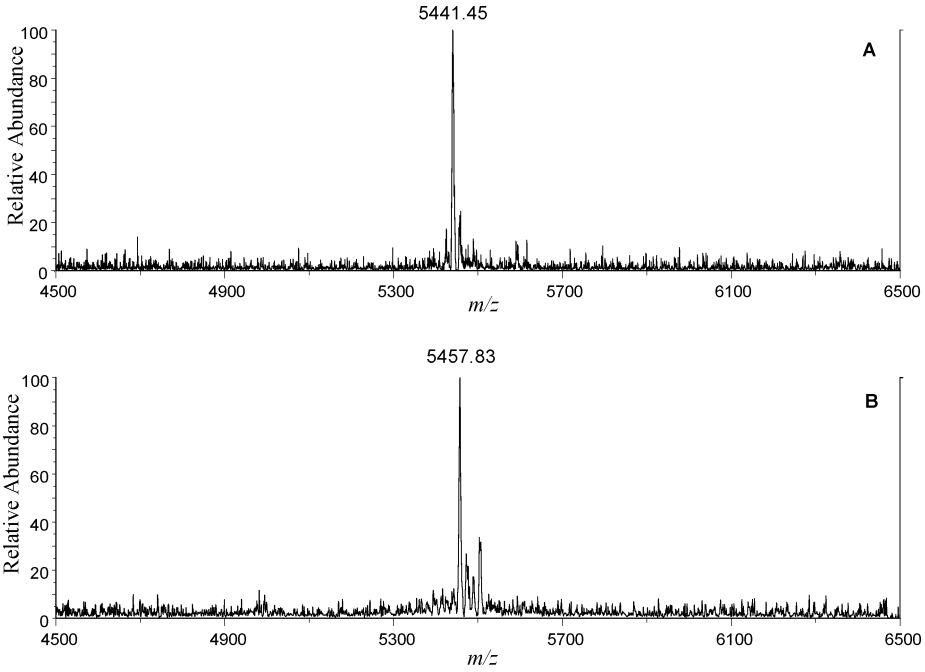

FIGURE 5: MALDI-TOF mass spectra of H_2O_2-modified and unmodified subunit VIIc. Panel A: MALDI-TOF mass spectrum analysis of RP-HPLC purified subunit VIIc isolated from unmodified CcO. Panel B: MALDI-TOF mass spectrum analysis of modified VIIc (VIIc* in Figure 3) isolated from CcO by RP-HPLC after reaction with 500 μM H_2O_2. The mass of the major peak at 5457.1 is 16 Da larger than that of unmodified VIIc. The minor peak at 5441.1 is due to the presence of some unmodified subunit VIIc.

Table 1: Collision-Induced Dissociation of Tryptic Peptide$_{10-20}$ of Control and Peroxide-Treated Cytochrome *c* Oxidase Subunit VIIc

C-terminal fragments (m/z)[a]						N-terminal fragments (m/z)[a]				
control[b]		+O[c]				control[b]			+O[c]	
calcd	obsd	calcd[d]	obsd		residue[e]		calcd	obsd	calcd[d]	obsd
					N_{10}	b_1	115.1		115.1	
1275.7		1291.7		y_{10}	I_{11}	b_2	228.1		228.1	228.0
1162.6	1162.4	1178.6	1178.3	y_9	P_{12}	b_3	325.2		325.2	
1065.5	1065.6	1081.5	1081.6	y_8	F_{13}	b_4	472.3		472.3	
918.5	918.3	934.5	934.4	y_7	S_{14}	b_5	559.3		559.3	
831.4	831.5	847.4	847.0	y_6	V_{15}	b_6	658.4		658.4	658.2
732.4	732.9	748.4	748.1	y_5	E_{16}	b_7	787.4	787.1	787.4	786.9
603.3	603.6	619.3	619.4	y_4	N_{17}	b_8	901.4	901.6	901.4	901.4
489.3	489.2	505.3	505.2	y_3	K_{18}	b_9	1029.5	1030.1	1029.5	1029.3
361.2	361.1	377.2	377.2	y_2	***W***$_{19}$	b_{10}	1215.6		1231.6	
175.1		175.1		y_1	R_{20}					

[a] Absence of an entry in the observed column indicates that the ion was not detected. [b] MS^2 fragmentation: m/z 695.4 →. [c] MS^2 fragmentation: m/z 703.3 →. [d] Fragment ions calculated for covalent attachment of O to Trp. [e] Numbering is based on the amino acid residue position in intact CcO subunit VIIc.

phenylalanines, and tryptophans. No indication of such channel was found for Trp48 within subunit IV, which may explain the lesser extent of Trp48 oxidation (~20%) in contrast with the ~80% oxidation of Trp19.

Oxidative damage to tryptophan has been detected in a number of cardiac mitochondrial proteins, including CcO subunit IV (*29*). In the latter case, doubly oxidized tryptophan occurred, and it was suggested that a small number of tryptophans might be "hot spots" for oxidative damage. Our experiments confirm the concept of hot spots within cytochrome *c* oxidase. Only 2 of the 22 tryptophans within the nuclear-encoded subunits of CcO are oxidized by hydrogen peroxide; i.e., 90% are completely resistant.

One intriguing aspect of our results is that similar oxidative damage may occur during normal enzymatic turnover *in situ*. Addition of hydrogen peroxide to purified cytochrome *c* oxidase produces the same catalytic intermediates that are transiently present during normal electron transport. If either the peroxy or the ferryl intermediate is the source of free radicals, then the only difference in our experiments is that hydrogen peroxide produces long-lived and static intermediates rather than short-lived, transient ones. Therefore, it is possible that similar free radical damage could naturally occur within the mitochondrion; for example, the Tyr244−His240 cross-link near the binuclear center (*30*) may be created by such a mechanism.

Identifying the direct cause of hydrogen peroxide-induced inactivation of cytochrome *c* oxidase, unfortunately, cannot be determined unambiguously. Oxidation of Trp19 within subunit VIIc (modified up to 70%) and dissociation of subunit VIIa (up to 60%) are the only changes that could account for the 70−80% decrease in enzymatic activity. Oxidized Trp48 within subunit IV or peroxidized cardiolipin are unlikely causative agents since less than 20% and 5% are formed, respectively. Although ~60% of subunit VIa dissociates during hydrogen peroxide-induced inactivation,

Table 2: Collision-Induced Dissociation of Tryptic Peptide$_{46-56}$ of Control and Peroxide-Treated Cytochrome *c* Oxidase Subunit IV

C-terminal fragments (m/z)[a]						N-terminal fragments (m/z)[a]				
control[b]		+O[c]				control[b]		+O[c]		
calcd	obsd	calcd[d]	obsd		residue[e]		calcd	obsd	calcd[d]	obsd
					A_{46}	b_1	72.0		72.0	
1151.6		1167.6		y_{10}	S_{47}	b_2	159.1		159.1	
1064.5	1064.4	1080.5	1080.3	y_9	***W***$_{48}$	b_3	345.2		361.2	361.0
878.4	878.2	878.4	878.2	y_8	S_{49}	b_4	432.2	432.0	448.2	448.1
791.4	791.2	791.4	791.2	y_7	S_{50}	b_5	519.2		535.2	535.1
704.4	704.2	704.4	704.2	y_6	L_{51}	b_6	632.3	632.1	648.3	648.3
591.3	591.2	591.3	591.2	y_5	S_{52}	b_7	719.3	719.3	735.3	735.0
504.3	504.4	504.3	504.4	y_4	I_{53}	b_8	832.4		848.4	848.3
391.2	391.0	391.2	391.1	y_3	D_{54}	b_9	947.4	947.3	963.4	963.2
276.2	276.0	276.2	276.3	y_2	E_{55}	b_{10}	1076.5	1076.0	1092.5	
147.1		147.1		y_1	K_{56}					

[a] Absence of an entry in the observed column indicates that the ion was not detected. [b] MS^2 fragmentation: m/z 611.8 →. [c] MS^2 fragmentation: m/z 619.8 →. [d] Fragment ions calculated for covalent attachment of O to Trp. [e] Numbering is based on the amino acid residue position in intact CcO subunit IV.

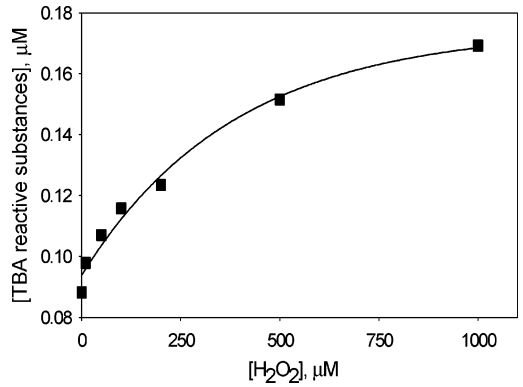

FIGURE 6: Peroxidation of CcO-associated cardiolipin. Cardiolipin peroxidation was determined by measurement of thiobarbituric acid (TBA) reactive substances using MDA as a standard. Cardiolipin was extracted from 5 μM CcO after it had been reacted with 0−1000 μM H_2O_2 for 30 min at room temperature. The fitted line is the nonlinear regression fit to a single exponential.

dissociation of this subunit cannot be responsible for CcO inactivation. Complete removal of subunit VIa is known to have only a minimal effect on the electron-transport activity of cytochrome *c* oxidase (*20*). The possibility that oxidation of a tryptophan or some other amino acid residue within one of the three core subunits inactivates CcO cannot be ruled out on the basis of the MALDI-TOF/MS analysis. It is not possible to detect an increase of only 16 or 32 mass units in subunits having a mass greater than 25 kDa.

Dissociation of subunit VIIa is the most likely cause of CcO inactivation. Subunit VIIa is located near the entrance to the D-channel, and loss of this subunit may hinder access of scalar protons into the binuclear center. Subunit VIIa is located close to subunit VIIc (*13*) and to one of the cardiolipins bound on cytochrome *c* oxidase (Sedlák and Robinson, unpublished results). Therefore, oxidation of Trp19 within subunit VIIc and/or cardiolipin bound at this site may induce dissociation of subunit VIIa. In support of this hypothesis, cytochrome *c* oxidase is inactivated when subunit VIIa is dissociated by urea, high hydrostatic pressure, or detergent (*17*; Sedlák, Staničova, Sowdal, and Robinson, unpublished results). Therefore, modification of subunit VIIc and dissociation of subunit VIIa are probably coupled events that lead to inactivation of cytochrome *c* oxidase.

In summary, when hydrogen peroxide reacts with the binuclear center, free radicals are generated and migrate relatively long distances to oxidatively damage specific tryptophans and cardiolipin. The final consequence is a 70−80% loss of enzymatic activity, oxidation of two tryptophans within subunits IV and VIIc, partial dissociation of subunits VIa and VIIa, and peroxidation of cardiolipin. Similar damage within the mitochondrion during normal enzymatic turnover is a distinct possibility.

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NOTE ADDED AFTER ASAP POSTING

This paper was inadvertantly published 01/08/04. The leftmost peak in Figure 3 is now correctly labeled. The corrected version was published 01/09/04.

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